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Flow Cytometric Analysis of Molt-Related Changes in Hemocyte Type in Male and Female *Penaeus japonicus*

TERESA SEQUEIRA[1], MANUEL VILANOVA[2], ALEXANDRE LOBO-DA-CUNHA[3], LUÍS BALDAIA[1], AND MÁRIO ARALA-CHAVES[2]

[1]*Laboratory of General Physiology,* [2]*Laboratory of Immunology,* [3]*Laboratory of Cellular Biology of the "Instituto de Ciências Biomédicas Abel Salazar," Porto, Portugal*

Abstract. Hemocyte cell suspensions obtained from male and female *Penaeus japonicus* were individually analyzed by flow cytometry through forward and side light-scatter parameters. The hemocyte cell suspensions were further characterized after cell sorting. This type of cell analysis has several advantages over microscopy techniques. After staining with phenoloxidase and peroxidase, the hemocytes were classified into the three classic categories of hyaline, semigranular, and granular cells. Significant cyclic differences were detected among the molting stages in both sexes. The hyaline cell population was predominant before and soon after the molt, decreasing over the intermolt. This decrease was, however, more prolonged in females. Thus, the hyaline cell population was dominant in stages B, D0, and D1 in males and only in stages B and D1 in females. Semigranular cells became predominant in females during the D0 stage.

Introduction

Most crustaceans molt throughout their lifetimes, and the periodic replacement of the cuticle is intrinsically linked with their physiology. Although their exoskeleton forms a structural and chemical barrier to parasites, they still need an efficient internal immune system to deal with microorganisms that might enter the hemocoel during ecdysis or through wounds, alimentary tract, or gills. This defense is largely based on the activities of the hemocytes (Söderhäll and Cerenius, 1992). Three types of circulating hemocytes can be distinguished on the basis of morphological criteria and different staining techniques (Bauchau, 1981), and were recently found to have different functions (Söderhäll *et al.*, 1990; Barracco *et al.*, 1991). It is known that hemocytes are affected by microorganisms (De Backer, 1961; Bang, 1971), but few data are available on hemocyte kinetics, particularly throughout the molt cycle (Bauchau and Plaquet, 1973; Tsing *et al.*, 1989). Moreover, the available morphological descriptions are not completely satisfactory because they are based on fixed cells or cells attached to an artificial substrate, resulting in a high percentage of unclassifiable cells.

Flow cytometry is a powerful method of cell analysis because quantitative multiparameter measurements on statistically large numbers of individual cells can be made without the necessity to pool cells from different individuals. Moreover, flow cytometry yields a large number of selected cells in a relatively short time. Furthermore, automation avoids much of the subjectivity inherent in microscopy. Therefore, flow cytometric analysis has been used in invertebrates to detect the DNA content evolution in nematodes (Hoshino *et al.*, 1991), molluscs (Elston *et al.*, 1990; Gerard *et al.*, 1994), insects (Marescalchi *et al.*, 1990), and insect cell lines (Odier *et al.*, 1993). This method has also been used to evaluate hemocyte proliferation upon antigenic stimulation in cockroaches (Ryan and Karp, 1993) and to investigate feeding behavior in molluscs through measurements of plankton cell size (Baldwin, 1991).

In this study, we used flow cytometry to analyze the hemocyte kinetics of a cultured shrimp, *Penaeus japonicus,* throughout its molt cycle.

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Address for correspondence: Prof. Mário Arala-Chaves, Laboratory of Immunology, Instituto de Ciências Biomédicas Abel Salazar, Largo Prof. Abel Salazar, 2, 4 050 Porto, Portugal.

Abbreviations. FSC, forward scatter; SSC, side scatter; H, hyaline; SG, semigranular; G, granular; proPO, prophenoloxidase activating system.

Materials and Methods

Shrimps

Penaeid shrimp for this study were bred in Eurodaqua, Algarve, Portugal. Males (71 animals, 15–25 *g*) and females (83 animals, 20–30 *g*) of *Penaeus japonicus* were maintained in a closed system tank at 12-h light/12-h dark, 3.5% salinity, and 20° ± 2°C and were fed a mixture of fresh mussels and squid three times a week. All shrimp were acclimated at least 1 month prior to use. The molting stages were determined according to Smith and Dall (1990).

Collection of hemolymph and preparation of cell suspensions

From each individual, 0.1–0.3 ml of hemolymph was collected by insertion of a needle syringe into the pericardial cavity. The hemolymph was directly withdrawn into the syringe containing 0.1 ml of anticoagulant buffer 0.2 *M* N-ethylmaleimide in 3% NaCl at 4°C (Martin *et al.*, 1991). Shrimp were bled at the same time of day to avoid possible variations, caused by endogenous rhythm, in the hemocyte populations. For cell size and density analyses, the hemolymph was mixed with 1 ml of 3.5% NaCl. Cells were washed (spun down at 167 × *g* for 10 min) and resuspended in 3.5% NaCl supplemented with 5% fetal calf serum. For cytochemistry, the hemolymph was drawn into a syringe containing 0.5 ml of 1% glutaraldehyde, 1% saccharose in sodium cacodylate buffer (0.2 *M*, pH 7.0). The cells were fixed for 15 min at 4°C, washed once in the cacodylate buffer and twice in the Tris-HCl buffer (0.1 *M*, pH 7.0) at 167 × *g* for 10 min.

Flow cytometry and sorting

Side- and forward-scatter parameters (SSC and FSC) were used for determination of cell granularity and cell size, respectively. SSC and FSC analyses were conducted in a FACScan analyzer (Becton-Dickinson, Mountain View, CA) with a Hewlett Packard computer (HP900) equipped with the LYSYS II analysis program (Becton Dickinson). As cells pass through the focused laser beam, light is scattered in all directions. The amount of light scattered at narrow angles to the axis (FSC parameter) of the laser beam is proportional to the cell size. The laser light scattered at right angles (SSC parameter) relates to the granularity or interior structure of the cell. Dead cells were excluded by propidium iodide (PI) incorporation. PI is a small molecule that binds to nucleic acids; it is very effectively excluded by cells with intact cell membranes, but dead cells become strongly fluorescent and thus are easily distinguished. At least 10,000 cells per sample were always analyzed. Cell sorting was performed in a FACSsort cell sorter (Becton-Dickinson). About 50,000 cells of each population were analyzed with the LYSYS II program.

Hemocyte cytochemistry

The presence of the prophenoloxidase system (proPO) in the hemocytes was determined by the method of Hose *et al.* (1987), incubating the cells in L-dopa (dihydroxyphenylalanine, 1 mg/ml in phosphate buffer 0.1 *M*, pH 7.4) for 16 h at room temperature and then examining them by light microscopy. The hemocyte peroxidase activity was determined by the method of Fahimi (1979). Thus the hemocytes were incubated for 3 h at 30°C in DAB (3,3-diaminobenzidine tetrahydrochloride in Tris-HCl buffer 0.1 *M*, pH 7.0) with 0.003% H_2O_2. The cells were examined by light microscopy. Control incubations were performed with 0.01 *M* sodium azide and 0.05 *M* triazol.

Results

Identification of hemocyte cell populations by flow cytometry

As shown in Figure 1, three hemocyte cell populations can be identified in individual male and female *P. japonicus* when FSC and SSC parameters are used to indicate different cell size and granularity. Hemocytes were analyzed during stages B, C, D0, and D1. After sorting and cytochemically staining each population and analyzing at least 150 cells per sample, it was possible to identify the three basic crustacean cell types. The cell population with low SSC and FSC parameters was considered to be of the hyaline (H) cell type because 98% ± 2% of these cells were both proPO and peroxidase negative (Fig. 2a, b). The cell population with the higher FSC and usually with an intermediate SSC parameter was considered to be semigranular (SG) because the cells were 92% ± 3% proPO positive and 85% ± 4% peroxidase negative (Fig. 2c, d). The cell population with the higher SSC and with FSC similar to H cells was considered to be of the granular (G) cell type. Indeed, strong phenoloxidase activity was detected in this cell population, which was 96% ± 4% positive for proPO and 83% ± 7% positive for peroxidase. Peroxidase activity was confined to the granules and was observed only in these cells (Fig. 2d, e). Furthermore, the intensity of this reaction could be decreased by incubation in sodium azide or triazole, as was reported by Lanz *et al.* (1993) (data not shown).

Changes in relative percentages of H, SG, and G cells during the molting cycle

As shown in Figure 3 and Table I, marked molt-related changes of the pool size of the three hemocyte cell pop-

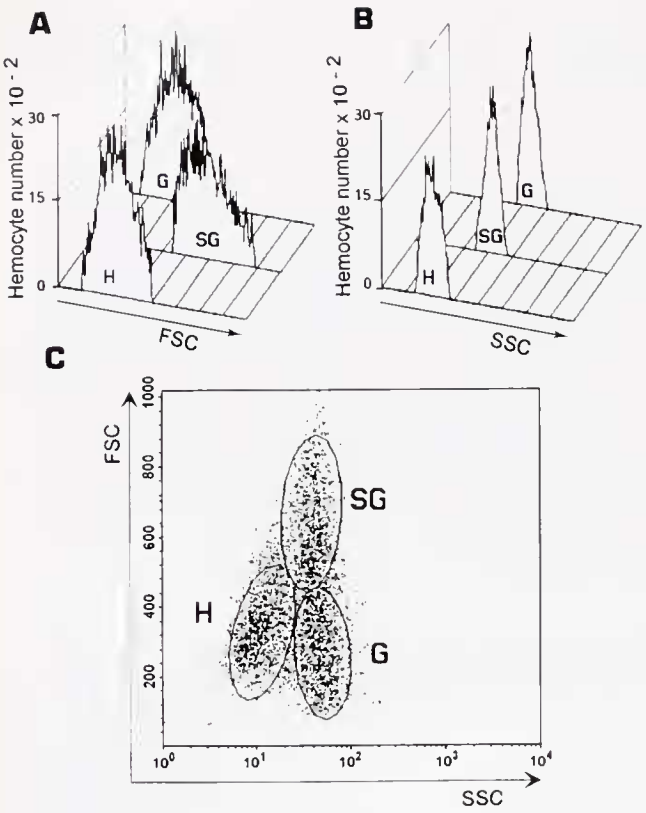

Figure 1. An example of flow cytometric analysis of fresh circulating hemocytes from *Penaeus japonicus* analyzed by forward scatter (FSC), indicating cell size; and side scatter (SSC), indicating cell granularity and structure. Frequency histograms of FSC (A), SSC (B), and a dot plot of both parameters (C) are shown. Hyaline, semigranular, and granular cell populations are designated H, SG, and G, respectively.

ulations were observed in both female and male *P. japonicus*. The patterns of distribution of the H, SG, and G cell populations are identical in both sexes during molt stages B and D1. The H cells are the most abundant (40%–44%), followed by the SG cells (31%–32%) with the G cells lowest (25%–29%). However, the percentages underwent a drastic change from stages B to C in cell type H, SG, and G in females, and in H and G cell types in males. These changes are still present over the D0 stage, but the pattern returned to that found in stage B, near the end of the cycle (stage D1). These intermolting changes, involving a decrease in the percentage of H cells and a subsequent increase in the percentages of SG and G cells, are more marked and more prolonged in females. Thus, the pattern of cell distribution observed in stage D0 is more similar to that observed in stage B in males than that in females. Moreover, the percentage of SG and of G cells has increased slightly from stage B to C to the same number as the H cells in females. In contrast, only the G cell population is increased slightly during the intermolting stage in males, and the number of SG cells is constant over the molting and intermolting stages.

Discussion

These flow cytometric results are in agreement with the general view that three circulating hemocyte populations are present in most crustaceans (Bauchau, 1981). Thus one of the three sorted cell populations totally lacked proPO and was composed of the smallest cells. The features of this cell population fit the H cell category described on the basis of microscopic techniques (Bauchau, 1981; Söderhäll and Smith, 1983; Lanz *et al.*, 1993). Phenoloxidase activity was present in the other two cell populations.

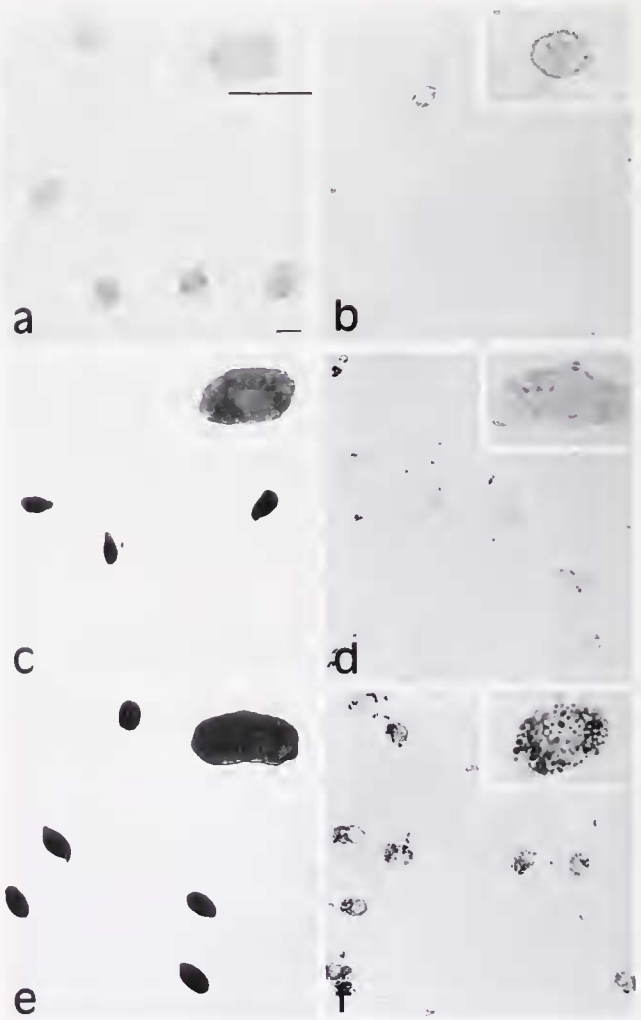

Figure 2. Light microscopy of fixed sorted cell populations stained with proPO (a, c, e) and with peroxidase (b, d, f) revealing hyaline (H) cells, proPO and peroxidase negative (a, b); semigranular (SG) cells, proPO positive and peroxidase negative (c, d); granular (G) cells, proPO and peroxidase positive (e, f). Bar = 10 μm.

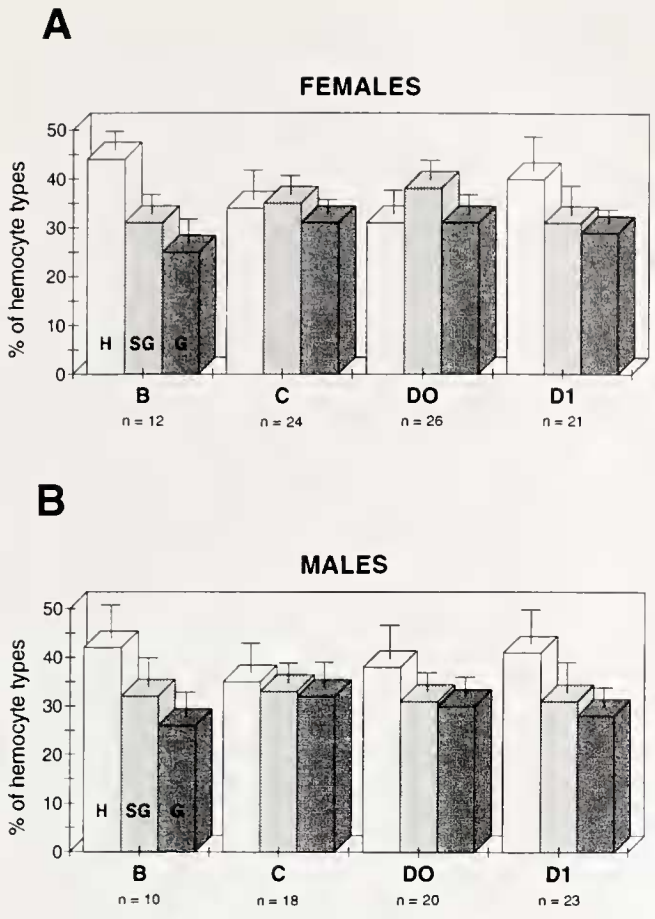

Figure 3. Frequencies of hyaline (H), semigranular (SG), and granular (G) hemocyte cell populations found in *Penaeus japonicus* hemolymph at the indicated stages of the molting cycle in females (A) and males (B). Analyses have been performed with 10,000 cells from each animal; *n* indicates the number of animals used for each value, which is presented as the mean and 1 standard deviation.

Cells of one of these populations were classified as G cells because their peroxidase activity was like that previously observed in similar cells in *Procambarus clarki* (Lanz *et al.*, 1993). When examined by light microscopy, the G cells appear slightly larger than the SG cells, but our flow cytometric results indicate that this may not be the case. This discrepancy might result from analyzing fresh cell suspensions instead of the fixed hemocyte smears used in light microscopy.

This study provides some evidence that the hemocyte cell populations of *P. japonicus* exhibit sex-related variations associated with the molt cycle. In both sexes, the relative percentages of H, SG, and G cells are identical after (stage B) and before (stage D1) ecdysis. Similar results were reported by Bauchau and Plaquet (1973). However, that study, which relied on morphological observations of hemocyte smears, was not directly comparable because SG and G cells could not be identified and were thus counted together, and because the samples were not identified by sex. More recently, Tsing *et al.* (1989) reported that no significant changes of the hemocyte cell populations occurred in *P. japonicus* during the molting stages. However, this study, like the one by Bauchau and Plaquet (1973), pooled male and female samples and was based on smear observations; furthermore, more than 50% of the hemocytes could not be identified. It seems, then, that flow cytometric analysis is a better and more reliable method for studying hemocyte variations than is the traditional technique of morphological observation of smears (Bauchau and Plaquet, 1973; Tsing *et al.*, 1989).

Molt-related changes in hemocyte populations were also demonstrated in insects (Crossley, 1965; Jones, 1967; Hinks and Arnold, 1977). The information about the role of molting hormones in these animals is not yet fully understood, but 20-hydroxyecdysone is known to induce a significant increase in the percentage of circulating phagocytic cells in *Calliphora erythrocephala* (Crossley, 1968). Although insects have somewhat different hemocyte types, the SG and G hemocytes, which may be phagocytic in crustaceans (Hose *et al.*, 1990), also increased in *P. japonicus* coincidentally with elevated ecdysteroid titers described in several crustaceans (Baldaia *et al.*, 1984; Roudy-Cuzin *et al.*, 1989). Tsing *et al.* (1989) also observed an increase in total hemocyte count during these molt stages. Ecdysteroid titers are higher and increase progressively from stages C to D1 in females. The rise of this hormone is considerably smaller in males and it is confined to the D0 stage (Baldaia *et al.*, 1984). Quanti-

Table I

Abundance of hemocyte types throughout the molting cycle of Penaeus japonicus

	H	SG	G
Females	B** > C = D0** < D1 = B	D0* > C* > B = D1	C = D0* > B = D1
Males	B* > C = D0 = D1 = B	B = C = D0 = D1	C = D0* > B = D1

Statistical analysis by paired Student's *t* test of the differences found in the hemocyte cell populations throughout the indicated molting stages. Differences are classified as nonsignificant at $P > 0.05$ (=); significant at $P < 0.05$ (*); and highly significant at $P < 0.01$ (**).

tative differences in ecdysteroids were also reported between the two sexes (Baldaia *et al.*, 1984; Roudy-Cuzin *et al.*, 1989); these may explain the sex-related differences reported here.

Flow cytometry is standardly used in mammalian hematology because it has large advantages over light microscopy. The present report indicates that this tool can also be used advantageously in the study of invertebrate cells such as hemocyte populations. In the future, such study may not only focus on cell size and granularity, but may also include more detailed analysis—for example, the detection of special cell markers using immunofluorescence-specific antibodies or the investigation of cell activation under various immunological stimuli.

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Literature Cited

Baldaia, L., P. Porcheron, J. Coimbra, and P. Cassier. 1984. Ecdysteroids in the shrimp *Palaemon serratus:* relation with molt cycle. *Gen. Comp. Endocrinol.* **55:** 437–443.

Baldwin, B. S. 1991. Ingestion of natural plankton by oyster larvae: the relative importance of different cell size fractions. *Am Zool.* **31:** 7A.

Bang, F. B. 1971. Transmissible disease, probably viral in origin, affecting the amoebocytes of the European shore crab *Carcinus maenas. Infect Immun* **3:** 617–623.

Barracco, M. A., B. Duvic, and K. Söderhäll. 1991. The β-1,3-glucan–binding protein from the crayfish, *Pacifastacus leniusculus* when reacted with a β-1,3-glucan, induces spreading and degranulation of crayfish granular cells. *Cell Tissue Res.* **266:** 491–497.

Bauchau, A. G., and J. C. Plaquet. 1973. Variation du nombre des hémocytes chez les crustacés brachyoures. *Crustaceana* **24:** 215–223.

Bauchau, A. G. 1981. Crustaceans. Pp. 386–420 in *Invertebrate Blood Cells.* Vol. 2. Academic Press, New York.

Crossley, A. C. 1965. Transformations in the abdominal muscles of the blue blow-fly *Calliphora erythrocephala* (Meig) during metamorphosis. *J Embryol. Exp. Morphol.* **14:** 375–398.

Crossley, A. C. 1968. The fine-structure and mechanism of break down of larval intersegmental muscles in the blowfly *Calliphora erythrocephala. J. Insect Physiol.* **14:** 1389–1407.

De Backer, J. 1961. Rôle joué par les hémocytes dans les réactions tissulaires de défense chez les crustacés. *Ann. Soc. R. Zool. Belgique* **92:** 141–151.

Elston, R. A., A. S. Drum, and S. K. Allen, Jr. 1990. Progressive development of circulating polyploid cells in *Mytilus* with hemic neoplasia. *Dis. Aquat. Org.* **8:** 51–59.

Fahimi, H. D. 1979. An assessment of the DAB methods for cytochemical detection of catalase and peroxidase. *J. Histochem. Cytochem.* **10:** 1365–1366.

Gerard, A., Y. Naciri, J. M. Peignon, C. Ledu, P. Phelipot, C. Noiret, I. Peudenier, and H. Grizel. 1994. Image analysis: a new method for estimation of triploidy in commercial bivalves. *Aquacult. Fish. Manage.* **25:** 697–708.

Hinks, C. F., and J. W. Arnold. 1977. Haematopoiesis in Lepidoptera. II. The role of haematopoietic organs. *Can. J. Zool.* **55:** 1740–1755.

Hose, J. E., G. G. Martin, V. A. Nguyen, J. Lucus, and T. Rosenstein. 1987. Cytochemical features of shrimp hemocytes. *Biol. Bull.* **173:** 178–187.

Hose, J. E., G. G. Martin, and A. S. Gerard. 1990. A decapod classification scheme integrating morphology, cytochemistry, and function. *Biol Bull.* **178:** 33–45.

Hoshino, K., K. Ohnishi, W. Yoshida, and T. Shinozawa. 1991. Analysis of ploidy in a planarian by flow cytometry. *Turbellarian Biol.* **227:** 175–178.

Jones, J. C. 1967. Effect of repeated haemolymph withdrawals and of ligaturing the head on differential counts of *Rhodnius prolixus* Stal. *J. Insect Physiol.* **13:** 1351–1360.

Lanz, H., V. Tsutsumi, and H. Aréchiga. 1993. Morphological and biochemical characterization of *Procambarus clarki* blood cells. *Dev. Comp. Immunol.* **17:** 389–397.

Marescalchi, O., V. Scali, and M. Zucotti. 1990. Genome size in parental and hybrid species of *Bacillus* (Insecta, Phasmatodea) from southeastern Sicily: A flow cytometric analysis. *Genome* **33:** 789–793.

Martin, G. G., J. E. Hose, S. Omori, C. Chong, T. Hoodbhoy, and N. McKrell. 1991. Localization and roles of coagulogen and transglutaminase in hemolymph coagulation in decapod crustaceans. *Comp. Biochem. Physiol* **100B:** 517–522.

Odier, F., P. Vago, J. M. Quiot, G. Devauchelle, and J. P. Bureau. 1993. Determination of DNA in densovirus-infected invertebrate cell line by flow cytometry. *J Invertebr Pathol.* **62:** 252–256.

Ryan, N. A., and R. D. Karp. 1993. Stimulation of hemocyte proliferation in the American cockroach (*Periplaneta americana*) by the injection of *Enterobacter cloacae. J Insect Physiol.* **39:** 601–608.

Roudy-Cuzin, J., C. Strambi, A. Strambi, and J.-P. Delbecque. 1989. Hemolymph ecdysteroids and molt cycle in males and females of *Siriella armata* M.-Edw. (Crustacea: Mysidacea): possible control by the MI-ME X organ of the eyestalk. *Gen. Comp. Endocrinol.* **74:** 96–109.

Smith, D. M., and W. Dall. 1990. Moult staging in the tiger prawn *Penaeus esculentus.* Second Australian National Prawn Seminar 85–93.

Söderhäll, K., and V. J. Smith. 1983. Separation of the haemocyte populations of *Carcinus maenas* and other marine decapods, and prophenoloxidase distribution. *Dev. Comp. Immunol.* **7:** 229–239.

Söderhäll, K., A. Aspán, and B. Duvic. 1990. The proPO-system and associated proteins; role in cellular communication in arthropods. *Res. Immunol.* **141:** 896–907.

Söderhäll, K., and L. Cerenius. 1992. Crustacean immunity. *Annu. Rev. Fish Dis.* **2:** 3–23.

Tsing, A., J.-M. Arcier, and M. Brehélin. 1989. Hemocytes of penaeid and palaemonid shrimps: morphology, cytochemistry, and hemograms. *J. Invertebr Pathol.* **53:** 64–67.